Exhibit A

Site-Directed Mutagenesis of Human Nucleoside Triphosphate Diphosphohydrolase 3: The Importance of Residues in the Apyrase Conserved Regions†

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Received November 28, 2000; Revised Manuscript Received February 2, 2001

ABSTRACT: Ecto-nucleoside triphosphate diphosphohydrolase 3 (eNTPDase-3, also known as HB6 and CD39L3) is a membrane-associated ecto-apyrase. Only a few functionally significant residues have been elucidated for this enzyme, as well as for the whole family of eNTPDase enzymes. Four highly conserved regions (apyrase conserved regions, ACRs) have been identified in all the members of eNTPDase family, suggesting their importance for biological activity. In an effort to identify those amino acids important for the catalytic activity of the eNTPDase family, as well as those residues mediating substrate specificity, 11 point mutations of 7 amino acid residues in ACR1−4 of eNTPDase-3 were constructed by site-directed mutagenesis. Mutagenesis of asparagine 191 to alanine (N191A), glutamine 226 to alanine (Q226A), and arginine 67 to glycine (R67G) resulted in an increase in the rates of hydrolysis of nucleoside diphosphates relative to triphosphates. Mutagenesis of arginine 146 to proline (R146P) essentially converted the eNTPDase-3 ecto-apyrase to an ecto-ATPase (eNTPDase-2), mainly by decreasing the hydrolysis rates for nucleoside diphosphates. The Q226A mutant exhibited a change in the divalent cation requirement for nucleotidase activity relative to the wild-type and the other mutants. Mutation of glutamate 182 to aspartate (E182D) or glutamine (E182Q), and mutation of serine 224 to alanine (S224A) completely abolished enzymatic activity. We conclude that the residues corresponding to eNTPDase-3 glutamate 182 in ACR3 and serine 224 in ACR4 are essential for the enzymatic activity of eNTPDases in general, and that arginine 67, arginine 146, asparagine 191, and glutamine 226 are important for determining substrate specificity for human ecto-nucleoside triphosphate diphosphohydrolase 3.

The ecto-nucleoside triphosphate diphosphohydrolase (eNTPDase)[1] family of enzymes (*1*), previously called E-type ATPases (*2*), hydrolyze a variety of nucleoside 5′-triphosphates and 5′-diphosphates in the extracellular space, and the relative hydrolysis rates for nucleoside triphosphates versus diphosphates vary considerably between enzymes in this family (*1*, *2*). The enzymes in this family consist of membrane-associated and soluble forms (*1*, *2*), and are expressed on a variety of cell types including endothelial cells (*3*), activated lymphocytes (*4*), skeletal and smooth muscle (*5*−*7*), and several types of tumors (*8*−*10*). Enzymatic activities of all eNTPDases are dependent on divalent cations such as calcium and magnesium.

The active site has not been delineated for any member of the eNTPDase family. It is also unclear why the family members differ in their substrate specificity. For example, eNTPDase-2 (ecto-ATPase) exhibits a high preference for the hydrolysis of nucleoside triphosphates versus diphosphates (NTPase:NDPase hydrolysis ratios greater than 25:1), while eNTPDase-1 (ecto-apyrase, CD39) hydrolyzes nucleoside di- and triphosphate almost equally well (ATPase:ADPase ratio ≈ 1). The relative rates of NTP versus NDP hydrolysis by eNTPDase-3 (also known as CD39L3 and HB6, ATPase:ADPase ratio = 3−4) are intermediate between eNTPDase-1 and eNTPDase-2 (*11*).

By multiple sequence alignments, four highly conserved regions, defined as apyrase conserved regions (ACRs), were found in all members of the eNTPDase family, from plants (*12*) to parasites (*13*) to mammals (*14*). Analysis of these ACRs revealed that certain motifs are also conserved in functionally important regions of proteins that belong to the actin/hsp70/sugar kinase family (*12*), a finding supported by mutagenesis studies of the "DXG" phosphate binding domains in ACR1 and ACR4 of eNTPDase-3 (*15*), as well as by mutagenesis of ACR residues in a man-made soluble

† This work was supported by Grants 96013960 and 9951504V from the American Heart Association, and by NIH Grant HL59915 (all to T.L.K.).

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; IDP, inosine 5′-diphosphate; ITP, inosine 5′-triphosphate; CDP, cytosine 5′-diphosphate; CTP, cytosine 5′-triphosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; NTPase, nucleoside triphosphohydrolase; NDPase, nucleoside diphosphohydrolase; NP40, Nonidet P-40 detergent; cDNA, complementary DNA; CD39, lymphoid cell activation antigen (eNTPDase-1); DSS, disuccinimidyl suberate; eNTPDase, ecto-nucleoside triphosphate diphosphohydrolase; HB6, human brain E-type ATPase clone (eNTPDase-3); MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi002711f CCC: $20.00
Published on Web 03/08/2001

form of eNTPDase-1 [CD39 (*16*)]. Thus, amino acid residues present in the ACRs are likely to be involved in functionally catalytic site(s) or be determinants for binding and/or hydrolysis properties modulating the differential ability of family members to hydrolyze NTPs versus NDPs. This nucleotide specificity is very likely to be involved in determining the different physiological functions for the members of the eNTPDase family.

By using chemical modification reagents, some classes of amino acids have been suggested to be important for eNTPDase function. Modification of arginines can reduce ATPase and ADPase activity 70−83% in rat brain ecto-apyrase (*17*). Modification of carboxyl groups of glutamate or aspartate decreases both ATPase and ADPase activity of the same ecto-apyrase (*17*). Interestingly, this inhibition can be prevented by preincubation with ATP and ADP or with GTP and GDP. Therefore, aspartic acid and/or glutamic acid residues are likely to be important for enzymatic activity, and we have previously demonstrated the importance of aspartic acids in ACR1 and ACR4 (*15*). Histidine residues have been implicated as important for eNTPDase activity by inactivation of nucleotidase activity by diethyl pyrocarbonate [DEPC (*6, 18*)]. Serine, tyrosine, histidine, and lysine residues have also been implicated as important for eNTPDase activity by chemical modification studies (*19*).

In this paper, we characterized the mutants generated from an arginine and conserved glutamate, asparagine, serine, and glutamine residues of ACR1−4 in eNTPDase-3. We found that glutamate 182 and serine 224 are required for the enzymatic function, while asparagine 191, glutamine 226, arginine 67, and arginine 146 are important residues for determining di- versus triphosphate substrate specificity.

MATERIALS AND METHODS

Materials. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. Oligonucleotides were synthesized by the DNA Core Facility at the University of Cincinnati. Lipofectamine Plus Reagent, Dulbecco's modified Eagle's medium (DMEM), calf serum, goat anti-rabbit horseradish peroxidase conjugated secondary antibody, and antibiotics/antimycotics were all obtained from Gibco/Life Technologies. The mammalian expression vector pcDNA3 was obtained from Invitrogen. Sequenase Version 2.0 kits were obtained from US Biochemical Corp., and ^{35}S-labeled dATP was from Dupont NEN. The chemical cross-linking reagent disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Co. Cibacron Blue Gel (Affi-Gel Blue) was obtained from BIO-RAD Laboratories. Ampicillin, nucleotides, and other reagents were from Sigma. Chymotrypsin A_4 was purchased from Boehringer Mannheim GmbH.

Site-Directed Mutagenesis of Human Brain eNTPDase-3 Ecto-apyrase. eNTPDase-3 (HB6) ecto-apyrase cDNA was isolated as described previously (*11*). Mutagenesis of the wild-type eNTPDase-3 in pcDNA3 vector was performed by using the QuikChange site-directed mutagenesis kit as described previously (*20*). The sense oligonucleotides used for mutagenesis are as follows with the substitution sites underlined:

R67G: 5′-CTGGATGCCGGGTCTTCAGGA-ACCACAGTCTACGTG-3′

R143A: 5′-GCCACGGCTGGGATGGCC-TTGCTGAGGTTGC-3′

R143K: 5′-GGAGCCACGGCTGGGATGAAG-TTGCTGAGGTTGC-3′

R146N: 5′-GCGCTTGCTGAAT-TTGCAAAATGAAAC-3′

R146P: 5′-GATGCGCTTGCTGCCG-TTGCAAAATGAAAC-3′

R146T: 5′-GATGCGCTTGCTGACG-TTGCAAAATGAAAC-3′

E182D: 5′-CATTTCTGGGCAACAAGAC-GGGGTATATGGATGG-3′

E182Q: 5′-CATTTCTGGGCAAGAACAA-GGGGTATATGGATGG-3′

N191A: 5′-GGATGGATTACAGCCGCC-TATTTAATGGGAAATTTC-3′

S224A: 5′-GGACTTAGGTGGTGCCGCC-ACCCAAATATCCTTCG-3′

Q226A: 5′-GGTGGTGCCTCCACCGCA-ATATCCTTCGTGG-3′

The complementary antisense oligonucleotides also necessary for the mutagenesis are not shown. The presence of the correct mutation and the lack of any unwanted mutations were confirmed by DNA sequencing using Sequenase version 2.0, and/or submitting the cDNA for fluorescent dye automated sequencing to the Department of Molecular Genetics in the College of Medicine at the University of Cincinnati.

Transient Transfection. COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and antibiotics and antimycotics, and transfected using Lipofectamine Plus Reagent as described previously (*21*). Briefly, 50−80% confluent cells in 100 mm dishes were transfected with either 4 μg of wild-type eNTPDase-3, 4 μg of mutagenized eNTPDase-3, or 4 μg of empty pcDNA3 vector for a background control. Approximately 48 h post-transfection, the COS-1 cells were harvested and used for analyses as described previously (*21*).

Total Membrane Preparation. Total cell membrane preparation was described previously (*11*). In short, cell monolayers were washed 3 times with ice-cold isotonic wash buffer containing 20 mM MOPS−NaOH, pH 7.4, 140 mM NaCl, and 5 mM KCl, harvested by scraping the culture dishes in tissue homogenization buffer (THB) containing 30 mM MOPS−NaOH, pH 7.4, 250 mM sucrose, 2 mM EDTA, and homogenized. The cell homogenate was centrifuged at 150000*g* for 60 min at 4 °C. The pellet was homogenized in a small volume of THB and used for functional assays.

Protein Assay. Protein concentrations were determined using the Bio-Rad CB-250 dye binding technique according to the modifications of Stoscheck (*22, 23*), using bovine serum albumin as the standard.

Nucleotidase Assays. Nucleotidase activities were determined by measuring the amount of inorganic phosphate (P_i) released from nucleotide substrates at 37 °C using a

modification of the technique of Fiske and Subbarow (*24*), as described previously (*6*). For cation-dependent nucleotidase activity, either 5 mM $MgCl_2$ or 5 mM $CaCl_2$ was used. The values were corrected for pcDNA3/COS cell background (COS cells transfected with an empty vector), as well as differences in expression levels as determined by Western blotting of each mutation. The units used for enzyme activity are micromoles of P_i per milligram of protein per hour.

Polyclonal Antibody Production. The anti-peptide polyclonal antiserum used in this study was raised against a peptide having the sequence of the extreme carboxy terminus of the eNTPDase ecto-apyrase (KRHSEHAFDHAVDSD-COOH), and was described previously (*21*).

Western Blot Analysis. Aliquots (0.5−5 μg) of membrane proteins, depending on the experimental purpose, were resolved in a 4−15% linear gradient SDS−polyacrylamide gel. Anti-eNTPDase-3 polyclonal antibody was used at a 1:2000 dilution. Immunoreactivity was detected by chemiluminescence reagents as described previously (*21*). Quantitation of the resultant X-ray film protein bands was accomplished using the AlphaImager 2000 documentation & analysis system (Alpha Innotech Corp., San Leandro, CA). Densitometry results are reported in Table 2, expressed as a percentage of the wild-type eNTPDase-3 protein level.

Cibacron Blue Affinity Purification. Cell membranes were solubilized with 1% NP-40 in 20 mM MOPS and 2 mM $MgCl_2$ (pH 7.4) at room temperature for 10 min. The solubilization mixture was centrifuged at 13000*g* for 3 min, and filtered through a 0.45 μm filter to remove any insoluble proteins. The filtrates were diluted 10-fold into 0.1% NP-40, 20 mM MOPS, and 2 mM $MgCl_2$ (pH 7.4) containing 20 μL of Cibacron Blue gel slurry, and incubated at room temperature on a rotator for 15 min. The Cibacron Blue gel beads containing the bound proteins were then washed with the above buffer 3 times and twice with 50 mM Tris-HCl (pH 6.8). Bound proteins were removed by boiling the gel slurry for 5 min in reducing (30 mM DTT) Laemmli sample buffer. SDS−PAGE and Western blotting were conducted as described above.

Cross-Linking Experiments. Membrane proteins (0.1 mg/mL) were incubated for 10 min at room temperature in 20 mM MOPS (pH 7.4) and 5 mM $MgCl_2$ in the presence or absence of a 100 μM sample of the hydrophobic, lysine-specific, primary amine cross-linker disuccinimidyl suberate (DSS), freshly prepared in dry DMSO. The lysine cross-linking reaction was stopped by adding an excess of lysine (10 mM) and incubating 5 min at room temperature. SDS−PAGE and Western blotting were performed as described above.

Limited Proteolysis Experiments. Membrane proteins at 0.2 mg/mL were incubated without or with 0.004 mg/mL chymotrypsin (1:50 ratio of protease to membrane protein) for 7 min at 22 °C in 50 mM Tris-HCl, pH 8.0, as has been described earlier (*20*). An equal volume of reducing SDS−PAGE sample buffer was added, and the samples were boiled for 5 min prior to SDS−PAGE and Western analysis as described above.

RESULTS

Expression and Enzymatic Activities of Wild-Type and Mutated Human eNTPase-3 Ecto-apyrase. Table 1 shows the sequences of the apyrase conserved sites 1−4 (ACR1−4) of the eNTPDase-3 enzyme used in this study, below the consensus sequences for ACR1−4 for the family of eNTPDases. The amino acids mutated in this work are double underlined in Table 1, and the amino acid substitutions that were made at each position are included, below the residue number of the mutated eNTPDase-3 amino acid. The level of protein expression as well as the expression level corrected ATPase and ADPase activities in the presence of 5 mM $MgCl_2$ for all of the mutations made is summarized in Table 2. Several mutations resulted in expression levels substantially below that of the wild-type, with the lowest expression of the R143K mutant, which was expressed at only 8% of the wild-type level, but was fully enzymatically active.

Table 1: eNTPDase-3 Apyrase Conserved Region (ACR) 1−4 Sequences, Showing the Amino Acid Residues Mutated in This Study (Double Underlined)[a]

Consensus sequence:		YGIXXDAGSXXTXXXXY	
ACR1 of eNTPDase-3	57	YGIVLDAGSSRTTVYVY	73
		67 G	
Consensus sequence:		TPXXLXXTAGXRXLX	
ACR2 of eNTPDase-3	132	TPIHLGATAGMRLLR	146
		143 146 A/K N/P/T	
Consensus sequence:		GXXEGXXXWXTXNX	
ACR3 of eNTPDase-3	179	GQEEGVYGWITANY	192
		182 D/Q 191 A	
Consensus sequence:		TXGXXDXGGXSTQXXF	
ACR4 of eNTPDase-3	214	TTGALDLGGASTQISF	229
		224 A 226 A	

[a] The consensus eNTPDase sequences are given above the ACR region sequences for the eNTPDase-3 enzyme used in this study. The residue numbers of the mutated amino acids in eNTPDase-3 are indicated in boldface directly under the ACR sequences, and the amino acid(s) to which these residues were mutated is (are) indicated in boldface under the mutated residue numbers. The "DXG" putative phosphate binding domains, similar to the actin superfamily phosphate binding domains, are underlined in ACR1 and ACR4 (*15*). The tryptophan residue (W) in ACR3 found to be essential for activity (*20*) is also underlined.

Table 2: Summary of All eNTPDase-3 Mutations Described in This Work[a]

ACR region of eNTPDase-3 mutation	mutation	expression level (wt % control)	ATPase act.	ADPase act.	ATPase: ADPase ratio
–	wt eNTPDase-3	100	72.3	19.9	3.6
ACR1	R67G	40	113	56.4	2.0
ACR2	R143A	35	27.4	11.1	2.5
ACR2	R143K	8	93.0	24.9	3.7
ACR2	R146N	95	75.5	15.9	4.7
ACR2	R146P	27	93.1	13.2	7.0
ACR2	R146T	89	103	27.5	3.7
ACR3	E182D	19	–	–	–
ACR3	E182Q	69	–	–	–
ACR3	N191A	45	23.8	26.9	0.88
ACR4	S224A	75	–	–	–
ACR4	Q226A	74	16.2	8.8	1.8

[a] All activities are in micromoles of P_i released per milligram of protein per hour in the presence of 5 mM magnesium, and have been corrected for both COS cell background activities as well as normalized for expression levels (relative to the wild-type enzyme). Dashes (−) indicate the absence of any detectable nucleotidase activity.

Mutation of the same amino acid residue to different amino acids resulted in substantially different expression levels in some cases. For example, E182Q was expressed at about 70% of the wild-type level, while E182D was expressed at only 19% of the wild-type level. All mutants studied in this report showed a lower expression level of protein compared to wild-type (Table 2). To test whether the lower expression level was due to differences in transcription of the mutated cDNAs, we examined the mRNA amount by RT-PCR in several mutants including R143A, E182D, N191A, and S224A. The results (data not shown) showed that there were no differences in mRNA levels between any of the mutants examined and the wild-type. Therefore, these point mutations do not generate their effects on protein expression levels via changes in transcription levels or the stability of the resultant mRNA.

The results in Table 2 showed that the E182D, E182Q, and S224A mutants completely lost all ATPase and ADPase activity. In the case of E182D, even the conservative substitution of aspartic acid for glutamic acid resulted in a complete loss of enzymatic activity. This indicates that glutamate 182 in ACR3 is crucial for the protein to maintain function and most likely no amino acid substitutions at that residue will maintain activity. The enzymatic activities of the inactive E182D, E182Q, and S224A mutations were also tested with different substrates, such as GTP, GDP, CTP, CDP, UTP, and UDP, and no activity was detected with any nucleotide (data not shown).

Both ATPase and ADPase activities were decreased in the R143A and Q226A mutants, and the percentage of wild-type activities was 38% and 22% for ATPase and 56% and 44% for ADPase, respectively. For the N191A mutant, the ATPase activity was decreased, and the ADPase activity was higher than that of wild-type. In three different amino acid substitution mutants of arginine 146, R146N had similar activities to wild-type, and R146T had higher activities than wild-type. Interestingly, R146P had higher ATPase activity and lower ADPase activity than the wild-type enzyme.

Nucleotide Hydrolysis Ratios and Substrate Preference for the Wild-Type eNTPDase-3 and Mutants. The relative rates of hydrolysis of ATP and ADP (the ATPase:ADPase ratio) for all the mutants are given in Table 2. The N191A, Q226A, and R67G mutations had lower ATPase:ADPase ratios (0.88, 1.8, and 2.0, respectively), while the R146P had a higher ratio (7.0), as compared to the wild-type eNTPDase-3 (3.6). To show that this effect was not specific for adenosine nucleotides, the hydrolysis of other nucleoside tri- and diphosphates was measured. Figure 1 shows that wild-type eNTPDase-3 ecto-apyrase and also the R67G, R146P, N191A, and Q226A mutants were all able to hydrolyze a broad range of nucleoside tri- and diphosphates. The trends of the change of NTPase:NDPase ratios for these four mutants utilizing all five different nucleoside tri- and diphosphates were similar (Figure 2). However, these mutants change the NTPase:NDPase ratios in different ways. Although both the NTPase and NDPase activities of the R67G mutant were increased, the increase of NDPase activity was greater than the increase in NTPase activity. The NTPase:NDPase ratios for all five purine and pyrimidine substrates were lower for R67G than for the wild-type enzyme.

The R146P mutation increased the NTPase:NDPase ratios by decreasing the NDPase activities, while nearly maintaining

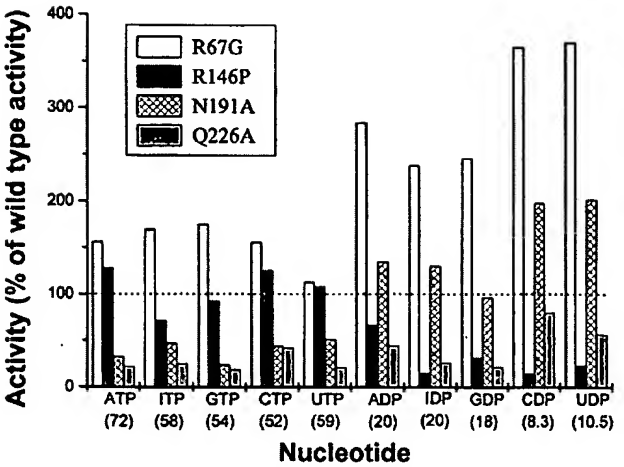

FIGURE 1: Nucleoside tri- and diphosphatase activity of the R67G, R146P, N191A, and Q226A mutants. Nucleotidase activities of wild-type eNTPDase-3 and mutants were determined as described under Materials and Methods. The specific activity (in micromoles of P_i per milligram of protein per hour, $n = 3$) of the wild-type eNTPDase-3 for each nucleotide is indicated in parentheses below each respective nucleotide. ATP was the preferred nucleotide substrate for the wild-type among these nucleotides. The enzymatic activities of four mutants are shown as a percentage of wild-type activity.

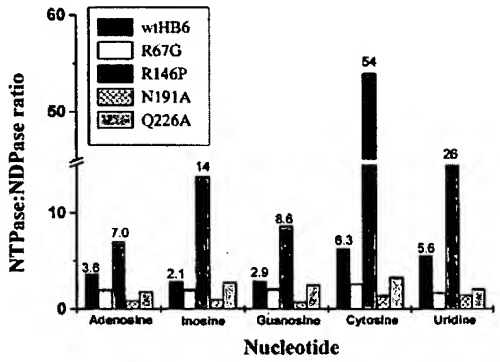

FIGURE 2: Nucleotidase ratio (NTPase:NDPase) of wild-type eNTPDase-3 and mutants using several purine (adenosine, inosine, and guanosine) and pyrimidine (cytosine and uridine) nucleotides. The enzymatic activities were assayed and calculated as described under Materials and Methods. In comparison with the wild-type, the R67G, N191A, and Q226A mutations decreased the NTPase:NDPase ratios for all nucleotides, while the R146P mutation increased the NTPase:NDPase ratios for all nucleotides, but especially evident for the inosine and cytosine nucleotides, for which the NTPase:NDPase ratios were increased 6.7- and 8.6-fold, respectively.

the wild-type NTPase level. Most strikingly, for the inosine and cytosine nucleotides, the NTPase:NDPase ratios for the R146P mutant are increased 6.7- and 8.6-fold as compared to wild-type, respectively. R146P showed higher preference for the nucleoside triphosphates than diphosphates, especially the pyrimidines CTP and UTP, for which the NTPase:NDPase ratios increased to 54:1 and 26:1, respectively. Thus, the replacement of arginine 146 by a proline residue partially converts the eNTPDase-3 ecto-apyrase to an ecto-ATPase (eNTPDase-2).

The decrease of NTPase:NDPase ratio observed for the N191A mutant was due to a decrease in the NTPase activity and an increase in the NDPase activity. The Q226A mutation resulted in both NTPase and NDPase activities being diminished, but the decrease of NTPase activity was more

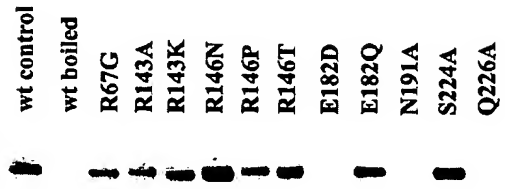

FIGURE 3: Cibacron Blue affinity purification of wild-type eNTPDase-3 and mutated enzymes. The wild-type eNTPDase-3 was used as a positive control, and the boiled wild-type (denatured protein) served as the negative control. Three micrograms of total membrane protein of the COS cell-expressed wild-type was used in this experiment, and the amount of each mutant used was normalized for the expression level to yield equivalent amounts of immunoreactive material used for all samples.

dramatic than NDPase activity. These results indicate that the N191A and Q226A mutations make the enzyme activity of eNTPDase-3 more similar to that observed for the CD39 ecto-apyrase (eNTPDase-1), which has an ATPase:ADPase ratio close to unity.

Conformational Analysis of Wild-Type eNTPDase-3 and Mutants. Cibacron Blue, a reactive nucleotide analogue, has been previously shown to bind to and inhibit the enzymatic activity of several E-type ATPases including eNTPDase-3 ecto-apyrase (*20, 25, 26*). Therefore, Cibacron Blue affinity purification was used to investigate whether the loss of enzymatic activities of several of the mutations in this study might be due to more global conformational changes that result in the loss of the ability to bind to Cibacron Blue affinity matrixes. As shown in Figure 3, most of the mutants retained the wild-type ability to bind to Cibacron Blue. However, the E182D, N191A, and Q226A mutants did not bind to the triazine dye matrix, indicating in these mutants the possibility of some tertiary or quaternary structural perturbation, leading to an abnormally folded protein, incapable of binding to the dye matrix. Although both the E182D and E182Q mutants completely lost enzymatic activities, E182D showed some delocalized changes in the conformation which E182Q does not, as judged by the inability of the E182D mutant to bind to Cibacron Blue. Therefore, these two mutants of the same glutamate residue may be inactivated by different mechanisms. The presumed delocalized conformational changes measured by Cibacron Blue binding were also observed in two other mutants, N191A and Q226A, which exhibited decreased activity as well as NTPase:NDPase ratios. Also evident from Figure 3, the loss of enzymatic activity of the S224A mutant is not due to delocalized conformational changes, since this totally inactive mutant bound to the Cibacron Blue matrix.

To further assess the possibility that point mutations introduced delocalized conformational effects on nucleotidase activities, a chemical cross-linker was used to test the ability of monomers to be cross-linked into oligomers. We have previously shown that the monomeric 79 kDa eNTPase-3 can be cross-linked to dimers of 158 kDa (and higher order oligomers) by a lysine-specific cross-linker, disuccinimidyl suberate (DSS) (*21*). The results of chemical cross-linking of wild-type and mutant eNTPDases are shown in Figure 4. Only mutations exhibiting substantial changes in nucleotidase activity profiles are shown; thus, the R143A, R143K, R146N, and R146T mutants are not included in this figure, since they

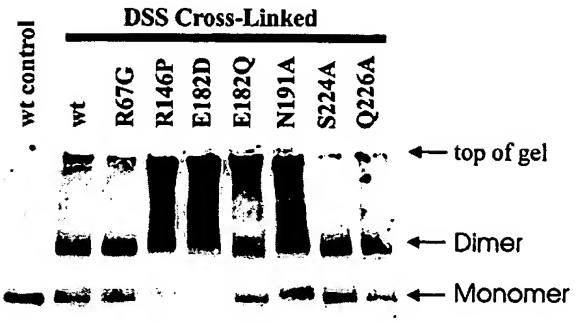

FIGURE 4: Western blot of 100 μM DSS cross-linked wild-type and mutant eNTPDase-3 cell membrane proteins. As described under Materials and Methods, 0.5 μg of wild-type total membrane protein was applied to a reducing SDS–PAGE gel, and immunoreactive equivalent amounts of the mutant enzymes (as determined by relative expression levels measured on Western blots) were applied to the same gel. The "wt control" lane is the wild-type enzyme without DSS cross-linking as a negative control for oligomer formation (all mutations appear as a 79 kDa band on reducing gels without cross-linking). The 79 kDa wild-type monomer was shown to shift to a 158 kDa dimer in the presence of DSS.

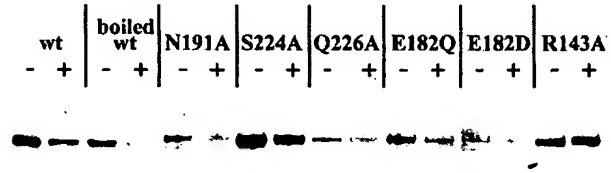

FIGURE 5: Limited chymotryptic digestion of wild-type and low-activity eNTPDase-3 mutants. Limited proteolysis was performed at a 1:50 protease to membrane protein (substrate) ratio for 7 min at 22 °C as described under Materials and Methods. Only the 79 kDa region of the resultant Western blot is shown, since no bands at other molecular masses were observed.

behaved similarly to the wild-type enzyme with respect to cross-linking (data not shown). The very active R67G mutant, the totally inactive S224A mutant, and the partially inactive Q226A mutant also behaved similarly to the wild-type enzyme with respect to cross-linking (Figure 4). Even the inactive E182Q and poorly active N191A mutants gave cross-linking results somewhat similar to the wild-type, although there is an increased formation of oligomers visible at the top of the gel. In contrast, nearly all the mutated eNTPDase-3 proteins from the active R146P and inactive E182D mutants formed dimers and higher oligomers upon cross-linking, indicating a change in the tertiary and/or quaternary structure of these mutants relative to wild-type. Importantly, both Cibacron Blue affinity binding and DSS cross-linking showed clear differences between the wild-type enzyme and E182D mutant. The Q226A mutant showed only the alteration in Cibacron Blue binding, and the R146P mutant showed only the alteration in DSS cross-linking.

Another approach used to assess the possibility that some of the point mutations mediated reduction in nucleotidase activity via a delocalized change in protein structure was limited chymotryptic digestion. The six mutations with the lowest nucleotidase activities (see Table 2) were tested for their susceptibility to limited proteolysis (Figure 5). Although clearly the wild-type conformation of the enzyme was partially digested under the conditions used, the denatured (boiled) wild-type and also the E182D, N191A, and Q226A

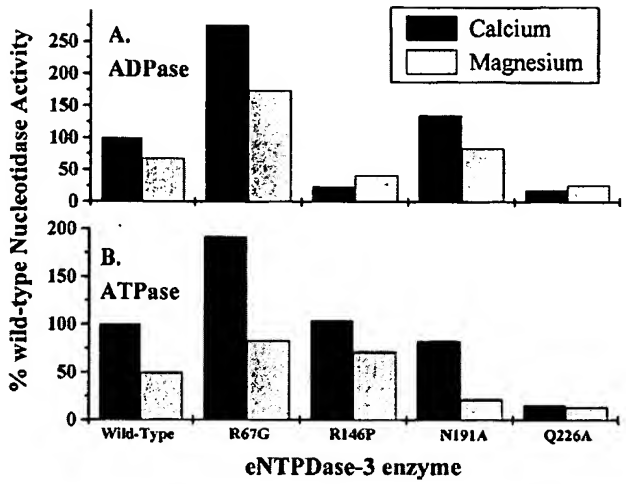

FIGURE 6: Divalent cation specificity of wild-type eNTPDase-3 and selected mutants. The assays for the enzymatic activity were performed in the presence of 2.5 mM adenine nucleotide with either 5 mM $CaCl_2$ or 5 mM $MgCl_2$, as described under Materials and Methods. The enzymatic activity of the wild-type enzyme in the presence of $CaCl_2$ was set to 100%. Panel A (top) shows the dependence of ADPase activity on divalent cation. Panel B (bottom) shows the ATPase activities assayed in the presence of calcium and magnesium.

mutants were almost totally digested under the same conditions, suggesting a difference in the conformational structure. This finding is consistent with the Cibacron Blue binding data (Figure 3), as well as the DSS cross-linking data (Figure 4), showing clear differences between these three mutants and the wild-type enzyme.

Divalent Cation Specificity of Wild-Type eNTPDase-3 and Selected Mutants. To investigate whether the divalent cation requirement of the nucleotidase activity of the mutant enzymes was altered, wild-type eNTPDase-3 and four mutants that exhibited changes in the NTPase:NDPase ratio were assayed for nucleotidase activity in the presence of either 5 mM $CaCl_2$ or $MgCl_2$. Wild-type eNTPDase-3 displayed a preference for calcium over magnesium for both ADPase and ATPase (Figure 6), and in the presence of calcium, the enzymatic activities were increased 1.5- and 2.0-fold, respectively, over those measured in the presence of magnesium. In this respect, the R67G and N191A mutants were similar to the wild-type. For the N191A mutant, the ratios are increased 1.6- and 3.8-fold, respectively, similar to the wild-type for ADPase, but much greater than for the wild-type with respect to ATPase activity. The Q226A mutant did not show a preference for either calcium or magnesium. Interestingly, the R146P mutant displayed a different preference for divalent cation-dependent ATPase and ADPase activities. For ATPase activity, calcium increased the enzymatic activity about 1.5-fold compared to magnesium, similar to the wild-type. In contrast, magnesium increased ADPase activity 1.7-fold compared to calcium, distinguishing this mutant from the wild-type enzyme.

DISCUSSION

eNTPDase-3 ecto-apyrase (HB6) was originally identified from a human brain cDNA library and has been suggested to be an ancestral enzyme for both CD39 ecto-apyrase (eNTPDase-1) and ecto-ATPase (eNTPDase-2) by phylogenetic analysis (*11*). In this work, several functionally important residues within the four highly conserved regions, ACR1−4 (*12*), were identified by site-directed mutagenesis.

Inactive Mutants: E182D, E182Q, and S224A. Glutamate 182 in ACR3 and serine 224 in ACR4 were identified to be essential for eNTPDase-3 enzymatic activity. These mutants completely lost enzymatic activity with adenosine (Table 2) and other (data not shown) nucleosides. These findings are consistent with and extend previous results using a recombinant, soluble, modified eNTPDase-1 [CD39 (*16*)]. In that recent report, alanine substitution of the glutamate residue corresponding to E182 in eNTPDase-3 in ACR3 abolished ATPase and ADPase activity, and alanine substitution of the serine residue corresponding to S224 in eNTPDase-3 in ACR4 reduced ATPase activity by 88% and ADPase activity by 91% (*16*). In our studies, we mutated the conserved glutamic acid (E182 in eNTPDase-3) not to alanine, but to the more closely related amino acids aspartic acid and glutamine. Even the conservative substitution of aspartic acid for glutamic acid resulted in complete loss of activity, indicating that this conserved glutamate residue in ACR3 may be important for the activity of all of the eNTPDases, and that it is likely that this residue may not be functionally replaced by any other amino acid.

Cibacron Blue binding (Figure 3), DSS cross-linking (Figure 4), and limited proteolysis (Figure 5) revealed no differences between the S224A mutant and the wild-type enzyme, and the expression level of this mutant was similar to wild-type. Therefore, the loss of activity in the S224A mutant is most likely not due to a delocalized or global conformational change. In contrast, the E182D mutant did not bind to the Cibacron Blue nucleotide analogue affinity matrix (Figure 3), suggesting an alteration in its nucleotide binding pocket. In addition, this mutant also formed some higher order oligomers upon cross-linking with DSS which are not observed for the wild-type (Figure 4), and was more susceptible to limited proteolysis (Figure 5). These data indicate that the loss of nucleotidase activity in the E182D mutant could be due, at least in part, to conformational changes in tertiary and quaternary structure. The conformational alteration may also explain the lower expression level observed for this mutant, since we noted in a previous study that some poorly expressed eNTPDase-3 mutations may be poorly expressed due to improper folding, leading to lower protein levels due to higher susceptibility to limited proteolysis (*20*).

In contrast to the E182D mutant, the E182Q mutant protein behaved more like the wild-type enzyme with respect to the Cibacron Blue binding, DSS cross-linking, and limited proteolysis assays (Figures 3−5). This suggests no delocalized changes in the structure for the E182Q mutant, and also that the loss of the negative charge in the active site might be the mechanism for inactivation of this mutant, consistent with what was observed when the glutamate residue corresponding to the E182 in eNTPDase-3 (E174 in soluble CD39) was mutated to alanine, resulting in an inactive enzyme (*16*). Thus, it appears that these two mutations of Glu 182 in eNTPDase-3 may inactivate the protein via different mechanisms.

Mutants with Altered Nucleoside Tri- versus Diphosphatase Activities: N191A, R146P, R67G, and Q226A. The mutation of asparagine 191 in ACR3 to alanine (N191A) caused a change of the ATP:ADP hydrolysis ratio from 3.6

(wild-type) to 0.88 by increasing the ADPase activity by about 135% and decreasing the ATPase activity by about 67%, relative to wild-type (Table 2). The ATPase:ADPase ratio observed for the N191A mutant was similar to that of the CD39 ecto-apyrase (eNTPDase-1), where the ATPase:ADPase ratio is 1.2:1. To extend and confirm the observation that, relative to the wild-type, the N191A mutant has increased ability to hydrolyze nucleoside diphosphates and decreased ability to hydrolyze nucleoside triphosphates, other purine substrates (ITP, IDP, GTP, and GDP) and pyrimidine substrates (CTP, CDP, UTP, and UDP) were tested. The results for the N191A mutant (Figure 1) showed the same trends for all five different nucleoside tri- and diphosphate pairs. Notably, this mutation resulted in about a 2-fold increase of CDPase and UDPase activity. Lack of binding to Cibacron Blue (Figure 3), increased formation of higher order oligomers after DSS cross-linking relative to wild-type (Figure 4), and increased susceptibility to limited proteolysis (Figure 5) all suggest that the alteration in the N191A mutant might be due to delocalized changes in the tertiary and quaternary structure of this mutant.

Of the three mutations incorporating different amino acid substitutions for Arg 146 in ACR2 (R146N/P/T), only R146P showed substantial differences from the wild-type enzyme. The mutations made were chosen due to the differences in the different eNTPDases enzyme sequences at this position. This residue is an arginine in the two eNTPDase-3 sequences as well as the CD39 (eNTPDase-1) sequences, but an asparagine residue in mammalian ecto-ATPases (eNTPDase-2), a threonine residue in the chicken ecto-ATPase (eNTPDase-2), and a proline in the soluble eNTPDase-5 and eNTPDase-6 enzymes. Thus, we hypothesized that this residue may have an effect on the nucleoside tri- versus diphosphatase activity for the eNTPDases, and made the appropriate mutations to test this hypothesis. The large increase of the NTPase:NDPase ratio for the R146P mutant (Table 2) seems to bear this out, especially for the pyrimidine (cytosine and uridine) nucleotides (Figure 2). This single R146P mutation essentially converts this ecto-apyrase (eNTPDase-3) to an ecto-ATPase (eNTPDase-2), at least for the pyrimidine nucleotides, as judged by the NTPase:NDPase ratios of 54 and 26 for cytosine and uridine nucleotides, respectively (see Figure 2). DSS cross-linking of the R146P mutant revealed more efficient cross-linking into dimers and higher order oligomers than the wild-type enzyme (Figure 4), suggesting a modification of the quaternary structure.

Arg 67 in ACR1 of eNTPDase-3 is unique because all other membrane-bound eNTPDases have a histidine residue in that position (*18*). The soluble and intracellular eNTPDases (eNTPDase4−6) contain a glycine residue at this position. The enzymatic activity of the R67G mutant constructed in this work is higher than wild-type, especially for NDPase activity, resulting in a decreased NTPase:NDPase hydrolysis ratio (Figure 1). In an earlier study by Grinthal and Guidotti (*27*), glycine or serine substitution of the corresponding residue (histidine) in CD39 apyrase essentially converted the CD39 apyrase (eNTPDase-1) to an ADPase (*27*). Our results extend this observation by showing that the eNTPDase-3 R67G mutant has slightly increased NTPase activities, but a much larger increase of all NDPase activities (Figure 1), leading to substantial decreases in all of the NTPase:NDPase ratios compared to the wild-type enzyme (Figure 2). The R67G mutant behaves like the wild-type enzyme in both the Cibacron Blue assay (Figure 3) and the cross-linking assay (Figure 4), suggesting no major changes in the tertiary or quaternary structure.

Mutants with Changes in Divalent Cation Preferences: R146P and Q226A. To investigate whether certain interesting mutations changed the divalent cation requirements or preferences for enzymatic activity, the mutations made in this study that resulted in substantial differences in NTPase:NDPase ratios were also examined for their activities in the presence of either Ca^{2+} or Mg^{2+} (Figure 6). The R67G and N191A mutants, like the wild-type enzyme, all exhibited higher ADPase and ATPase activities in the presence of Ca^{2+}. The R146P and Q226A mutants showed different trends for the dependence of the activity on calcium and magnesium. For the R146P mutant, the ATPase activity was higher in the presence of calcium, like the wild-type, but the ADPase activity was higher in the presence of magnesium, differing from the wild-type enzyme. However, the Q226A mutant displayed nearly identical activity in the presence of either calcium or magnesium. Thus, these data suggest that arginine 146 in ACR2 and glutamine 226 in ACR4 may be involved in binding of the divalent cation or divalent cation−substrate complex.

In summary, we show that several conserved residues in the apyrase conserved regions 1−4 are important for eNTPDase-3 to maintain normal nucleotidase activity and to mediate substrate specificity. Glutamate 182 and serine 224 are essential for enzymatic activity. Arginine 67, arginine 146, asparagine 191, and glutamine 224 are important for modulation of the relative activity using NTPs versus NDPs as substrates. The E182D, N191A, and Q226A mutations all resulted in a loss of binding to the nucleotide analogue, Cibacron Blue, as well as changes in chemical cross-linking and susceptibility to limited proteolysis, suggesting a delocalized conformational change in these mutants which may account for their low nucleotidase activities.

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BI002711F